

OPI DATE 05/12/95 APPLN. ID 22841/95
AOJP DATE 13/01/96 PCT NUMBER PCT/US95/04426



INT.

AU9522841

(51) International Patent Classification ⁶ : C07H 15/12, C12N 15/00, A61K 49/00		A1	(11) International Publication Number: WG 95/31466
			(43) International Publication Date: 23 November 1995 (23.11.95)
(21) International Application Number: PCT/US95-04426		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GY, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
(22) International Filing Date: 10 April 1995 (10.04.95)			
(30) Priority Date: 08/242,188 13 May 1994 (13.05.94) US			
(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US).		Published <i>With international search report.</i>	
(72) Inventors: PRUSINER, Stanley, B.; 400 Pacheco Street, San Francisco, CA 94116 (US). SCOTT, Michael, R.; 1200 Clayton Street #9, San Francisco, CA 94114 (US). TELLING, Glenn; 1224 11th Avenue, San Francisco, CA 94122 (US).			
(74) Agent: BERLINER, Robert; Robbins, Berliner & Carson, 5th floor, 201 N. Figueroa Street, Los Angeles, CA 90012-2628 (US).			
(54) Title: METHOD OF DETECTING PRIONS IN A SAMPLE AND TRANSGENIC ANIMAL USED FOR SAME			
(57) Abstract The invention includes an artificial PrP gene, a transgenic animal containing the gene, and an assay wherein the transgenic animal is used to detect the presence of pathogenic prions in a sample or diagnose a cause of death.			

METHOD OF DETECTING PRIONS IN A SAMPLE
AND TRANSGENIC ANIMAL USED FOR SAME

Government Rights

The United States Government may have certain rights in this application pursuant to Grant Nos. NS14069, AG02132, NS22786, AG08967 and AG10770 awarded by the National Institutes of Health.

Field of the Invention

This invention relates generally to chimeric genes, methods of assaying and to transgenic animals used in such assays. More specifically, this invention relates to artificial and chimeric PrP genes, assaying samples for pathogenic prions, and to transgenic mice containing an artificial or chimeric PrP gene.

15

Background of the Invention

Prions are infectious pathogens that cause central nervous system spongiform encephalopathies in humans and animals. Prions are distinct from bacteria, viruses and viroids. The predominant hypothesis at present is that no nucleic acid component is necessary for infectivity of prion protein. Further, a prion which infects one species of animal (e.g., a human) will not infect another (e.g., a mouse).

25 A major step in the study of prions and the diseases that they cause was the discovery and purification of a protein designated prion protein ("PrP") [Bolton et al., Science 218:1309-11 (1982); Prusiner et al., Biochemistry 21:6942-50 (1982); McKinley et al., Cell 35:57-62 (1983)]. Complete prion protein-encoding genes have since been cloned, sequenced and expressed in transgenic animals. PrP^C is encoded by a single-copy host gene [Basler et al., Cell 46:417-28 (1986)] and is normally found at the outer surface of neurons. A leading hypothesis is that prion diseases result from conversion of PrP^C into a modified form called PrP^{Sc}. However, the

30

35

actual biological or physiological function of PrP^{sc} is not known.

It appears that the scrapie isoform of the prion protein (PrP^{sc}) is necessary for both the transmission and pathogenesis of the transmissible neurodegenerative diseases of animals and humans. See Prusiner, S.B., "Molecular biology of prion disease," Science 252:1515-1522 (1991). The most common prion diseases of animals are scrapie of sheep and goats and bovine spongiform encephalopathy (BSE) of cattle [Wilesmith, J. and Wells, Microbiol. Immunol. 172:21-38 (1991)]. Four prion diseases of humans have been identified: (1) kuru, (2) Creutzfeldt-Jakob Disease (CJD), (3) Gerstmann-Strassler-Scheinker Disease (GSS), and (4) fatal familial insomnia (FFI) [Gajdusek, D.C., Science 197:943-960 (1977); Medori, et al., N. Engl. J. Med. 326:444-449 (1992)]. The presentation of human prion diseases as sporadic, genetic and infectious illnesses initially posed a conundrum which has been explained by the cellular genetic origin of PrP.

Most CJD cases are sporadic, but about 10-15% are inherited as autosomal dominant disorders that are caused by mutations in the human PrP gene [Hsiao et al., Neurology 40:1820-1827 (1990); Goldfarb et al., Science 258:806-808 (1992); Kitamoto et al., Proc. R. Soc. Lond. (In press) (1994)]. Iatrogenic CJD has been caused by human growth hormone derived from cadaveric pituitaries as well as dura mater grafts [Brown et al. Lancet 340:24-27 (1992)]. Despite numerous attempts to link CJD to an infectious source such as the consumption of scrapie infected sheep meat, none has been identified to date [Harries-Jones et al., J. Neurol. Neurosurg. Psychiatry 51:1113-1119 (1988)] except in cases of iatrogenically induced disease. On the other hand, kuru, which for many decades devastated the Fore and neighboring tribes of the New Guinea highlands, is believed to have been spread by infection during ritualistic cannibalism [Alpers, M.P..

Slow Transmissible Diseases of the Nervous System, Vol. 1, S.B. Prusiner and W.J. Hadlow, eds. (New York: Academic Press), pp. 66-90 (1979)].

The initial transmission of CJD to experimental
5 primates has a rich history beginning with William
Hadlow's recognition of the similarity between kuru and
scrapie. In 1959, Hadlow suggested that extracts
prepared from patients dying of kuru be inoculated into
non-human primates and that the animals be observed for
10 disease that was predicted to occur after a prolonged
incubation period [Hadlow, W.J., Lancet 2:289-290
(1959)]. Seven years later, Gajdusek, Gibbs and Alpers
demonstrated the transmissibility of kuru to chimpanzees
after incubation periods ranging from 18 to 21 months
15 [Gajdusek et al., Nature 209:794-796 (1966)]. The
similarity of the neuropathology of kuru with that of CJD
[Klatzo et al., Lab Invest. 8:799-847 (1959)] prompted
similar experiments with chimpanzees and transmissions of
disease were reported in 1968 [Gibbs, Jr. et al., Science
20 161:388-389 (1968)]. Over the last 25 years, about 300
cases of CJD, kuru and GSS have been transmitted to a
variety of apes and monkeys.

The expense, scarcity and often perceived inhumanity
of such experiments have restricted this work and thus
25 limited the accumulation of knowledge. While the most
reliable transmission data has been said to emanate from
studies using non-human primates, many cases of human
prion disease have been transmitted to rodents but
apparently with less regularity [Gibbs, Jr. et al., Slow
30 Transmissible Diseases of the Nervous System, Vol. 2,
S.B. Prusiner and W.J. Hadlow, eds. (New York: Academic
Press), pp. 87-110 (1979); Tateishi et al., Prion
Diseases of Humans and Animals, Prusiner et al. eds.
(London: Ellis Horwood), pp. 129-134 (1992)].

35 The infrequent transmission of human prion disease
to rodents has been cited as an example of the "species
barrier" first described by Pattison in his studies of

passaging the scrapie agent between sheep and rodents [Pattison, I.H., NINDS Monograph 2, D.C. Gajdusek, C.J. Gibbs Jr. and M.P. Alpers, eds. (Washington, D.C.: U.S. Government Printing), pp. 249-257 (1965)]. In those
5 investigations, the initial passage of prions from one species to another was associated with a prolonged incubation time with only a few animals developing illness. Subsequent passage in the same species was characterized by all the animals becoming ill after
10 greatly shortening incubation times.

The molecular basis for the species barrier between Syrian hamster (SHa) and mouse was shown to reside in the sequence of the PrP gene using transgenic (Tg) mice [Scott et al., Cell 59:847-857 (1989)]. SHaPrP differs
15 from MoPrP at 16 positions out of 254 amino acid residues [Basler et al., Cell 16:417-428 (1986); Loch et al., Proc. Natl. Acad. Sci. USA 83:6372-6376 (1986)]. Tg(SHaPrP) mice expressing SHaPrP had abbreviated incubation times when inoculated with SHa prions. When
20 similar studies were performed with mice expressing the human, or ovine PrP transgenes, the species barrier was not abrogated, i.e., the percentage of animals which became infected were unacceptably low and the incubation times were unacceptably long. Thus, it has not been
25 possible to use transgenic animals (such as mice containing a PrP gene of another species) to reliably test a sample to determine if that sample is infected with prions. The seriousness of the health risk resulting from the lack of such a test is exemplified
30 below.

More than 45 young adults previously treated with HGH derived from human pituitaries have developed CJD [Koch et al., N. Engl. J. Med. 313:731-733 (1985); Brown et al. Lancet 340:24-27 (1992); Fradkin et al., JAMA
35 265:880-884 (1991); Buchanan et al., Br. Med. J. 302:824-828 (1991)]. Fortunately, recombinant HGH is now used, although the seemingly remote possibility has been raised

that increased expression of wtPrP^{Sc} stimulated by high HGH might induce prion disease [Lasmezas et al., Biochem. Biophys. Res. Commun. 196:1163-1169 (1993)]. That the HGH prepared from pituitaries was contaminated with prions is supported by the transmission of prion disease to a monkey 66 months after inoculation with a suspect lot of HGH [Gibbs, Jr. et al., N. Engl. J. Med. 328:358-359 (1993)]. The long incubation times associated with prion diseases will not reveal the full extent of iatrogenic CJD for decades in thousands of people treated with HGH worldwide. Iatrogenic CJD also appears to have developed in four infertile women treated with contaminated human pituitary-derived gonadotrophin hormone [Healy et al., Br. J. Med. 307:517-518 (1993); Cochius et al., Aust. N.Z. J. Med. 20:592-593 (1990); Cochius et al., J. Neurol. Neurosurg. Psychiatry 55:1094-1095 (1992)] as well as at least 11 patients receiving dura mater grafts [Nisbet et al., J. Am. Med. Assoc. 261:1118 (1989); Thadani et al., J. Neurosurg. 69:766-769 (1988); Willison et al., J. Neurosurg. Psychiatric 54:940 (1991); Brown et al. Lancet 340:24-27 (1992)]. These cases of iatrogenic CJD underscore the need for screening pharmaceuticals that might possibly be contaminated with prions.

Recently, two doctors in France were charged with involuntary manslaughter of a child who had been treated with growth hormones extracted from corpses. The child developed Creutzfeldt-Jakob Disease. (See New Scientist, July 31, 1993, page 4). According to the Pasteur Institute, since 1989 there have been 24 reported cases of CJD in young people who were treated with human growth hormone between 1983 and mid-1985. Fifteen of these children have died. It now appears as though hundreds of children in France have been treated with growth hormone extracted from dead bodies at the risk of developing CJD (see New Scientist, November 20, 1993, page 10.) In view of such, there clearly is a need for a convenient, cost-

effective assay for testing sample materials for the presence of prions which cause CJD. The present invention offers such an assay.

5 Summary of the Invention

The invention includes an artificial PrP gene, a transgenic animal containing the gene, and assay methodology which uses the transgenic animal to detect pathogenic prions in a sample. The artificial gene includes a sequence such that when it is inserted into the genome of an animal (such as a mouse), the animal is rendered susceptible to infection with prions which normally would infect only a specific species of genetically diverse animal (such as human, cow or sheep). The artificial PrP gene may be comprised of a completely artificial polynucleotide sequence. Alternatively, the artificial gene may be comprised of the codon sequence of a first animal with one or more codon substitutions being made wherein the substitutions are preferably corresponding PrP gene codons from a genetically diverse animal with the proviso that not all of the different codons are replaced by codons of the genetically diverse animal.

Pathogenic prions in a sample can be detected by injecting the sample to be tested into a transgenic mouse. In one preferred example the mouse genome includes a chimeric PrP gene which gene includes a portion of a gene of the animal (e.g. human) in danger of infection from prions in the sample. For example, Creutzfeldt Jakob Disease (CJD) is a fatal neurodegenerative disease of humans caused by prions. Preferred transgenic (Tg) mice disclosed herein express a chimeric prion protein (PrP) in which a segment of mouse (Mo) PrP was replaced with the corresponding human (Hu) PrP sequence. The chimeric PrP designated MHu2MPPrP, differs from MoPrP by 9 amino acids between residues 96 and 167. All of the Tg(MHu2MPPrP) mice injected with

human prions developed neurologic disease. More specifically, the transgenic mice of the invention developed the disease ~200 days after inoculation with brain homogenates from three CJD patients. When inoculated with CJD prions, MHu2MPrP^{Sc} was formed; in contrast MoPrP^{Sc} was produced if Mo prions were inoculated. The patterns of MHu2MPrP^{Sc} and MoPrP^{Sc} accumulation in the brains of Tg(MHu2M) mice were different. About 10% of Tg(HuPrP) mice expressing HuPrP and Non-Tg mice developed neurologic disease >500 days after inoculation with CJD prions. The different susceptibilities of Tg(HuPrP) and Tg(MHu2M) mice to human prions indicates that additional species specific factors are involved in prion replication. Tg(MHu2MPrP) mice disclosed herein are useful in the diagnosis, prevention and treatment of human prion diseases. Transgenic mice containing the chimeric prion protein gene which includes a portion, but not all, of the PrP gene of an animal from a different genus can be used to test samples for prions which might infect such animals. The transgenic mice disclosed herein consistently develop the adverse effects of such prions in a relatively short time and are substantially cheaper and easier to maintain than are currently used primate models.

25 An object of the invention is to provide a gene which may be artificial or chimeric which gene when inserted into the genome of one animal (e.g., a mouse) will render the mammal susceptible to infections from prions which naturally only infect a genetically diverse mammal, e.g., human, bovine or ovine.

30 Another object of the invention is to provide an assay for the detection of prions in a sample.

Another object is to provide an artificial PrP gene wherein one or more codons (preferably 1-50 codons) of the PrP gene of a first animal (e.g. a mouse) is replaced with codons of the PrP gene of a genetically diverse animal (e.g. a human, cow or sheep) in a manner so as to

render the first animal susceptible to infection with prions which normally infect only the genetically diverse animal.

Another object is to provide a chimeric gene
5 comprised of codons encoding the C- and N- terminus of one species of mammal and middle codons of another species of mammal.

Another object of the invention is to provide a transgenic mammal such as a mouse which includes a
10 chimeric PrP gene which gene includes a portion of the PrP gene from another animal, such as a human, cow, or sheep.

An advantage of the present invention is that the transgenic mouse can be used to assay for the presence of
15 prions in a sample in a manner which is substantially faster, more efficient and cheaper than presently available assay methods.

Another advantage is that transgenic mice inoculated with prions of humans can be used as test animals for
20 testing drugs for efficacy in the treatment of humans suffering from diseases resulting from infection with prions.

Another advantage is that the transgenic mice can detect prions in a sample at very low levels, e.g.,
25 1 part per million, and even as low as 1 part per billion.

Still another advantage is that the mice provide an assay which is highly accurate, i.e., does not provide false positives and consistently determines the presence
30 of prions.

Yet another advantage is that by increasing the copy number of the gene of the invention in a transgenic mammal, the incubation time for prion caused disease is decreased.

35 A feature of the present invention is that the transgenic mice injected with a sample containing pathogenic prions will consistently develop the disease

effects of the prions within a relatively short time, e.g. about 200 days \pm 50 days after injection or less.

Another feature is that an artificial gene of the invention preferably contains codons of the PrP gene of a first animal (such as a mouse) with some (but not all) of the codons which differ from the mouse and a second genetically diverse mammal (such as a human) replacing codons of the first mammal at the same relative positions.

These and other objects, advantages, and features of the invention will become apparent to those persons skilled in the art upon reading the details of the chimeric gene, assay method, and transgenic mouse as more fully described below.

15

Brief Description of the Drawings

Figure 1 is a schematic drawing showing the construction of a chimeric MHu2M gene and a transgenic mouse containing same;

Figure 2 is a schematic view of a portion of PrP proteins showing the differences between a normal, wild-type human PrP protein and a normal, wild-type mouse PrP protein;

Figure 3 shows the amino acid sequence of mice PrP along with specific differences between mice PrP and human PrP;

Figure 4 shows the amino acid sequence of mouse PrP and specifically shows differences between mouse PrP and bovine PrP; and

Figure 5 shows the amino acid sequence of mouse PrP and specifically shows differences between mouse PrP and ovine PrP.

Detailed Description of Preferred Embodiments

Before the present chimeric gene, assay methodology and transgenic mice used in the assay are described, it is to be understood that this invention is not limited to

particular assay methods, chimeric and artificial genes or transgenic mice described, as such methods, chimeric genes and mice may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

The term "prion" shall mean an infectious particle known to cause diseases (spongiform encephalopathies) in humans and animals. The term "prion" is a contraction of the words "protein" and "infection" and the particles are comprised largely if not exclusively of PrP^{Sc} molecules encoded by a PrP gene. Prions are distinct from bacteria, viruses and viroids. Known prions include those which infect animals to cause scrapie, a transmissible, degenerative disease of the nervous system of sheep and goats as well as bovine spongiform encephalopathies (BSE) or mad cow disease and feline spongiform encephalopathies of cats. Four prion diseases known to affect humans are (1) kuru, (2) Creutzfeldt-Jakob Disease (CJD), (3) Gerstmann-Strassler-Scheinker Disease (GSS), and (4) fatal familial insomnia (FFI). As used herein prion includes all forms of prions causing all or any of these diseases or others in any animals

used - and in particular in humans and in domesticated farm animals.

The term "PrP gene" refers generally to any gene of any species which encodes any form of a prion protein.

5 The term "artificial PrP gene" is used herein to encompass the term "chimeric PrP gene" as well as other recombinantly constructed genes which when included in the genome of one animal (e.g., a mouse) will render the mammal susceptible to infection from prions which
10 naturally only infect a genetically diverse mammal, e.g., human, bovine or ovine. In general, an artificial gene will include the codon sequence of the PrP gene of the mammal being genetically altered with one or more (but not all, and generally less than 50) codons of the
15 natural sequence being replaced with a different codon - preferably a corresponding codon of a genetically diverse mammal (such as a human). The genetically altered mammal being used to assay samples for prions which only infect the genetically diverse mammal. Examples of artificial
20 genes are mouse PrP genes encoding the sequence as shown in Figures 3, 4 and 5 with one or more different replacement codons selected from the codons shown in these Figures for humans, cows and sheep replacing mouse codons at the same position, with the proviso that not
25 all the mouse codons are replaced with differing human, cow or sheep codons.

The terms "chimeric gene," "chimeric PrP gene" and the like are used interchangeably herein to mean an
30 artificially constructed gene containing the codons of a first animal such as a mouse with one or more of the codons being replaced with corresponding codons from a genetically diverse animal such as a human, cow or sheep. In one specific example the chimeric gene is comprised of the starting and terminating sequence (i.e., N- and C-
35 terminal codons) of a PrP gene of a mammal of a first species (e.g. a mouse) and also containing a nucleotide sequence of a corresponding portion of a PrP gene of a

mammal of a second species (e.g. a human). A chimeric gene will, when inserted into the genome of a mammal of the first species, render the mammal susceptible to infection with prions which normally infect only mammals of the second species. The preferred chimeric gene disclosed herein is MHu2M which contains the starting and terminating sequence of a mouse PrP gene and a non-terminal sequence region which is replaced with a corresponding human sequence which differs from a mouse PrP gene in a manner such that the protein expressed thereby differs at nine residues.

The terms "susceptible to infection" and "susceptible to infection by prions" and the like are used interchangeably herein to describe a transgenic mammal of the invention which has an 80% or greater, preferably 98% or greater, and most preferably a 100% chance of developing a disease if inoculated with prions. The terms are used to describe a transgenic animal of the invention such as a transgenic mouse Tg(MHu2M) which, without the chimeric PrP gene, would not be susceptible to infection with a human prion (less than 20% chance of infection) but with the chimeric gene is susceptible to infection with human prions (80% to 100% chance of infection).

The term "incubation time" shall mean the time from inoculation of an animal with a prion until the time when the animal first develops detectable symptoms of disease resulting from the infection. A reduced incubation time is one year or less, preferable about 200 days \pm 50 days or less, more preferably about 50 days \pm 20 days or less.

Abbreviations used herein include CJD for Creutzfeldt-Jakob Disease; GSS for Gerstmann-Strassler-Scheinker Disease; FFI for fatal familial insomnia; PrP^{Sc} for the scrapie isoform of the prion protein; MoPrP for a mouse prion protein; SHa for a Syrian hamster; BSE for bovine spongiform encephalopathy; CNS for central nervous

- system; MHu2M for a chimeric mouse/human PrP gene wherein a region of the mouse PrP gene is replaced by a corresponding human sequence which differs from mouse PrP at 9 codons; Tg(MHu2M) mice are transgenic mice of the invention which include the MHu2M gene;
- CNS for central nervous system;
- CJD for Creutzfeldt-Jakob Disease;
- GSS for Gerstmann-Strassler-Scheinker Disease;
- FFI for fatal familial insomnia;
- 10 Hu for human;
- HuPrP for a human prion protein;
- Mo for mouse;
- SHaPrP for a Syrian hamster prion protein;
- Tg for transgenic;
- 15 Tg(SHaPrP) for a transgenic mouse containing the PrP gene of a Syrian hamster,
- Tg(HuPrP) for transgenic mice containing the complete human PrP gene;
- Tg(ShePrP) for transgenic mice containing the complete sheep PrP gene;
- 20 Tg(BovPrP) for transgenic mice containing the complete cow PrP gene;
- MoPrP^{sc} for the scrapie isoform of the mouse prion protein;
- 25 MHu2MPPrP^{sc} for a chimeric gene wherein a portion of the scrapie isoform of the human PrP gene is fused into the scrapie isoform of the mouse PrP gene,
- PrP^{CJD} for the CJD isoform of a PrP gene;
- Prn-p^{0/0} for ablation of both alleles of the MoPrP gene;
- 30 Tg(SHaPrP^{+/0})81/Prn-p^{0/0} for a particular line (81) of transgenic mice expressing SHaPrP, +/0 indicates heterozygous;

General Aspects of the Invention

- 35 The present invention includes several aspects including: (1) an artificial gene comprised of codon sequences which when inserted into the genome of a first

animal (e.g. a mouse or rat) will render the animal susceptible to infection with prions which normally infect only a genetically diverse animal (e.g. a human, cow or sheep), thereby including genes wherein 1 to 50
5 codons of a naturally occurring PrP gene of a first animal are replaced with corresponding codons of a genetically diverse animal; (2) a chimeric gene which gene is comprised of the PrP sequence of a gene of a mammal of a first species which gene has been modified to
10 include a corresponding segment of a PrP gene of a mammal of a second species; (3) a transgenic mammal containing an artificial or a chimeric gene of the invention such as a transgenic mouse including a chimeric PrP gene wherein a portion of the mouse gene is replaced with a
15 corresponding portion of a human PrP gene thereby rendering the mouse susceptible to infection with human prions; (4) a method of determining whether a sample is infected with prions which method involves inoculating a transgenic mammal of the invention with a sample to be
20 tested and observing the mammal for a period of time sufficient to determine if the mammal develops symptoms of a disease normally associated with prions; (5) a method of testing the efficacy of a drug in the treatment of disease developed as a result of infection with prions
25 comprising administering a drug to be tested to a transgenic animal infected with prions and observing and/or testing the mammal to determine if the drug aids in promoting or slowing the progress of the disease or the symptoms; and (6) a method for determining the cause
30 of death of an animal comprising inoculating a transgenic animal of the invention with extracted brain tissue from the animal which has died and observing the transgenic animal in order to determine if the transgenic animal develops symptoms of prion infections.

35 Preferred host animals are mice and rats, with mice being most preferred in that there exists considerable knowledge on the production of transgenic animals. Other

possible host animals include those belonging to a genus selected from *Mus*, *Rattus*, *Oryctolagus*, and *Mesocricetus*. The host PrP gene can include codon from genetically diverse PrP genes from animals belonging to a genus selected from *Bos*, *Ovis*, *Sus* and *Homo*. Preferably, a mouse host PrP gene is changed to include a codon from a human, cow or sheep PrP gene, with human being most preferred.

The genetic material which makes up the PrP gene is known for a number of different species of animals. Further, there is considerable homology between the PrP genes in different mammals. For example, see the amino acid sequence of mouse PrP compared to human, cow and sheep PrP in Figures 3, 4 and 5 wherein only the differences are shown. Further, note that the segment of a PrP gene used to create the MHu2M gene of the present invention will result in encoding of protein which shows a difference between the human and a mouse protein of only nine residues. Although there is considerable genetic homology with respect to PrP genes, the differences are significant in some instances. More specifically, due to small differences in the protein encoded by the PrP gene of different mammals, a prion which will infect one mammal (e.g. a human) will not normally infect a different mammal (e.g. a mouse). Due to this "species barrier", it is not generally possible to use animals such as mice as test animals in order to determine whether a particular sample contains prions which would normally infect a different species of animal such as a human. The present invention solves this problem in a surprising manner.

At first, it might appear that useful transgenic test animals could be produced by substituting the PrP gene of the animal which would act as the test animal (e.g., a mouse) with the PrP gene of a different animal (e.g., a human) and use the test animal to determine if it becomes infected with prions of the other animal. i.e.

prions which normally infect only a human. However, when such transgenic animals are produced and used as test animals, they are not readily infected with the prions which normally infect the mammal from which their transgenic gene was taken. Thus, artificial or chimeric genes of the invention will not have all of the host codons replaced with different codons from the genetically diverse animal. A more specific description of how the species barrier was broken in accordance with the present invention is provided below.

Species Barrier Broken

The transmission of human CJD to apes and monkeys 1.5 - 3 years after intracerebral inoculation provided considerable interest in the causes of neurodegenerative diseases [Gibbs, Jr. et al., Science 161:388-389 (1968)]. Humans are not genetically diverse from apes and monkeys which accounts for the cross-species infectivity, although with a long incubation time. While the high cost of caring for nonhuman primates prevented extensive studies of the human prion diseases, the transmissibility of these diseases stimulated studies of the animal prion analogues in rodents [Manuelidis et al., Proc. Natl. Acad. Sci. USA 75:3422-3436 (1978); Manuelidis et al., Proc. Natl. Acad. Sci. USA 73:223-227 (1976); Tateishi et al., Ann. Neurol. 5:581-584 (1979)].

The present disclosure opens a new frontier in the investigation of the human prion diseases since transmission studies can now be performed relatively rapidly in genetically altered mammals such as Tg(MHu2M) mice that are relatively inexpensive to maintain. For the first time, endpoint titrations of prions in multiple human body tissues and fluids can be performed and standard curves constructed for more economical incubation time assays. The information derived from such studies of human prions will be useful in the management of CJD patients who are thought to pose some

risk to relatives, physicians, nurses and clinical laboratory technicians [Berger et al., Neurology 43:205-206 (1993); Ridley et al., Lancet 341:641-642 (1993)].

In studies of human prion diseases with apes and monkeys, the use of one or two, or rarely three, animals as recipients for a single inoculum has presented a significant problem in evaluating the transmissibility of a particular inoculum from an individual patient. The Tg(MHu2M) mice described here obviate many of the problems created by using nonhuman primates. The results demonstrate the "universality" of the MHu2M transgene for transmission studies with other types of transgenic animals and other prion inocula. For example, it may be most efficient to use mice expressing MHu2MPrP transgenes coding for either a methionine or valine at codon 129, and by doing so, match the genotype of the Tg mouse (with respect to codon 129) with the genotype of the individual from which the inoculum is derived. Homozygosity at the codon 129 polymorphism has a profound influence on the incidence of sporadic CJD [Palmer et al., Nature 352:340-342 (1991)]. The MHu2MPrP transgene encodes a Met at codon 129 and the iatrogenic CJD case was homozygous for Met [Collinge et al., Lancet 337:1441-1442 (1991)].

To break the species barrier we have created an artificial PrP gene which, when inserted into a first mammal (such as a mouse) renders that mammal susceptible to infection with prions which normally infect only a genetically diverse mammal (e.g. a human, cow or sheep). The artificial PrP gene may include the natural PrP gene sequence of the first animal with one or more (preferably less than 50) codon sequences being replaced with other codon sequences such as corresponding codons of a genetically diverse mammal (e.g. a human, cow or sheep).

In a specific example of the invention the species barrier is broken by inserting into a mammal (a mouse) the chimeric gene (MHu2M) which chimeric gene which is shown being assembled schematically in Figure 1. In

order to produce the chimeric gene, it is first necessary to obtain nucleotide sequences which encode human PrP. The human PrP genes are then subjected to the conventional PCR procedures in order to produce large numbers of copies of the gene or portions of the gene. The PCR product is then isolated, specific restriction sites are added and the copied product is subjected to specific endonucleases in order to remove a middle section of the human PrP gene. Specifically, restriction sites are added such that when the PCR product is subjected to endonucleases such as Asp718 as well as BstEII, a section of the gene is cut out. The use of these two endonucleases will remove a center portion of the human PrP gene (codons 94-188) which portion encodes amino acid residues 94 through 188. Endonucleases are also used to remove a corresponding center portion of the mouse PrP gene. The removed center portion of the mouse gene is then discarded and the center portion obtained from the human PrP gene is fused into the mouse gene to produce a chimeric human/mouse gene. Details of how the specific MHu2M gene was produced are described in Example 1 and shown in Figure 1.

As shown with Figure 2, there is a high degree of homology between the removed center portion of the human PrP gene and the segment of the mouse PrP gene which is replaced. Specifically, the segments differ at nine codons. Thus, when the genetic material is expressed, the resulting chimeric MHu2M protein will differ from MoPrP at 9 residues. These residues and their positions are shown in Figure 2. After the chimeric gene is produced, it can be microinjected into a mouse egg using known technology as described within Scott et al., Cell 59:847-857 (1989) and Scott et al., Protein Sci. 1:986-997 (1992) and see also WO91/19810 published December 22, 1991 as well as other publications relating to the production of transgenic mice cited therein and known to those skilled in the art. The injected mouse egg is then

implanted into a mouse using known procedures. Multiple eggs can be implanted into a single mouse and known procedures can be used to determine whether the resulting offspring are transgenic mice which include the chimeric gene within their genome. Details of this procedure are described in Example 1.

It is not possible to render a mammal susceptible to infection with prions from a genetically diverse mammal by substituting, completely, the PrP gene of the mammal with the complete PrP gene of the genetically diverse mammal. We have successfully broken the "species barrier" by producing a chimeric PrP gene wherein a middle portion of the mouse PrP gene is replaced with a corresponding middle portion of a human PrP gene thereby leaving the C- and N-terminus of the mouse PrP gene intact. However, other segments of the mouse PrP gene can be replaced with other homologous segments of the human PrP gene and obtain a transgenic mouse which is subject to being readily infected with human prions. Thus, the invention is not limited to the particular chimeric gene MHu2M or chimeric mice produced using this gene. The invention includes all types of transgenic animals which include artificial genes wherein the artificial gene renders the transgenic animal susceptible to infection with prions which normally infect only a genetically diverse animal.

Numerous specific examples of artificial genes of the invention can be deduced from reviewing Figures 3, 4 and 5. Specifically, one may start with the basic PrP gene of a mouse (as the first animal) which animal is to become the transgenic animal. Thereafter, one or more codons of the mouse gene may be replaced with one or more corresponding codons of a human, bovine or sheep PrP gene which codon is different from the corresponding codon of the mouse gene but at the same relative position in the gene. By showing that it is possible to break the "species barrier" by creating a particular chimeric gene

whereby transgenic mice can test for the presence of human prions we have opened the door for the creation of other transgenic animals which will include other artificial PrP genes which, for example, can allow for the testing for the presence of bovine or ovine prions in a sample.

Tg(MHu2M) mice with shorter incubation times

The incubation time of Tg(MHu2M) mice inoculated with Hu prions is now about 200 days \pm 50 days. We believe this can be reduced substantially. In Tg(SHaPrP) mice, the level of SHaPrP transgene expression was found to be inversely proportional to the length of the scrapie incubation time after inoculation with SHa prions [Prusiner et al., Cell 63:673-686 (1990)]. Thus, producing Tg(MHu2M) mice with higher levels of transgene expression should substantially reduce the incubation times.

Based on other studies which we have performed using an analogous hamster/mouse chimeric PrP gene, MH2M, it is possible to assign a theoretical optimal incubation period for the MHu2M construct in a mouse lacking the endogenous mouse PrP gene. We obtained incubation periods of ~105 days with a heterologous Syrian hamster prion inoculum, shortening to ~62 days with a homologous MH2M prion inoculum. The shortest incubation period so far observed in any of our transgenic mouse studies was ~45 days for a line expressing the mouse PrP gene. Assuming a similar minimum incubation period with MHu2M prions in Tg(MHu2M PrP) mice lacking the endogenous mouse PrP gene, we can confidentially expect incubation periods of the order of $45 \times 105/62 = 76$ days with human prions. This is a conservative estimate, even shorter incubation periods may be possible in lines with very high copy numbers. Copy numbers can be increased up to 300-400 and the incubation timer can be as short as 50 days \pm 20 days. In addition, other chimeric human/mouse PrP

constructs may exhibit even shorter incubation times than MHu2M PrP.

In addition, removing MoPrP^C by crossing Tg(MHu2M) mice onto an ablated background (Prn-p^{0/0}) may also reduce the incubation time since Tg(SHaPrP^{+/0})81/Prn-p^{0/0} mice exhibit a 30% reduction in incubation times compared to Tg(SHaPrP^{+/0})81/Prn-p^{+/+} mice [Büeler et al., Cell 73:1339-1347 (1993). Prusiner, S.B. et. al., Proc. Natl. Acad. Sci. USA 90:10608-10612 Nov. 1993. Accordingly, we have also used fertilized eggs from mice in which the endogenous PrP gene has been ablated as recipients for microinjection of the MHu2M PrP construct.

By systematically altering the extent and position of the substitutions in other chimeric Hu/Mo PrP constructs, it is possible to further enhance the susceptibility of Tg mice to Hu prions as reflected by shortened incubation times. Shortening the incubation time is a worthwhile goal for the facilitation of many future studies in prion research and for the evaluation of pharmaceuticals.

In general, there is an inverse relationship between the number of copies of a chimeric or artificial PrP gene and the incubation time of disease after inoculation of the transgenic animal with prions. Specific 2Hu2M mice disclosed herein have only 3 or 4 copies of the 2Hu2M gene. The number of copies can be increased to 3 to 4 hundred thereby significantly reducing the incubation time from about 200 days to 50 days \pm 20 days.

30 Differences in the conversion of MHu2MPrP^C and HuPrP^C into the scrapie isoform in mice

The fundamental event in prion propagation seems to be the conversion of PrP^C, which contains ~43% α -helix and is devoid of β -sheet, into PrP^{Sc} which has ~43% β -sheet [Pan et al., Proc. Natl. Acad. Sci. USA 90:10962-10966 (1993)]. From the results of Tg(SHaPrP) mouse studies, this process is thought to involve the formation of a

complex between PrP^{Sc} and the homotypic substrate PrP [Prusiner et al., Cell 63:673-686 (1990)]. Attempts to mix PrP^{Sc} with PrP^C have failed to produce nascent PrP^{Sc} [Raeber et al., J. Virol. 66:6155-6163 (1992)], raising
5 the possibility that proteins such as chaperones might be involved in catalyzing the conformational changes that feature in the formation of PrP^{Sc}. One explanation for the difference in susceptibility of Tg(MHu2M) and Tg(HuPrP) mice to Hu prions in mice may be that mouse
10 chaperones catalyzing the refolding of PrP^C into PrP^{Sc} can recognize MHu2MPrP but not HuPrP.

Another possibility is that sequences at the N- or C-terminus of HuPrP inhibit the formation of PrP^{Sc} in murine cells. To test this possibility, HuPrP sequences
15 are substituted for the Mo sequences at each terminus of MHu2MPrP. Comparison of the PrP sequences in many mammals around the GPI anchor addition site (codons 227-235) reveals an interesting difference of four amino acids between rodents and primates [Stahl et al.,
20 Biochemistry 31:5043-5053 (1992)]. In support of this hypothesis is that rodents also differ from ruminants including sheep and cattle at this site; sheep prions have failed to transmit neurodegeneration to Tg(ShePrP). In these experiments the entire mouse PrP gene was
25 replaced with the entire sheep PrP gene.

In contrast to Tg(MHu2M) mice, the overall transmission rate of Hu prion inocula from a wide variety of sources was less than 10% in Tg(HuPrP) mice, no different from the rate observed in non-Tg mice.
30 Likewise the conversion of HuPrP^C into HuPrP^{Sc} in Tg(HuPrP) mice appears to be a relatively infrequent event similar to the rare conversion of MoPrP^C to PrP^{Sc} in response to human prions. The low rates of transmission in these mice do not seem to be a consequence of low titers of
35 human prion titers: two inocula which failed to cause disease in Tg(HuPrP) mice transmitted to 100% of inoculated Tg(MHu2M) animals.

"Strains" of human prions

Studies in rodents have shown that prion strains produce different patterns of PrP^{Sc} accumulation [Hecker et al., Genes & Development 6:1213-1228 (1992); DeArmond et al., Proc. Natl. Acad. Sci. USA 90:6449-6453 (1993)]; which can be dramatically changed by the sequence of PrP^{Sc} [Carlson et al., Proc. Natl. Acad. Sci. USA in press (1994)]. The molecular basis of prion diversity has for many years been attributed to a scrapie specific nucleic acid [Bruce et al., J. Gen. Virol. 68:79-89 (1987)] but none has been found [Meyer et al., J. Gen. Virol. 72:37-49 (1991); Kellings et al., J. Gen. Virol. 73:1025-1029 (1992)]. Other hypotheses to explain prion strains include variations in PrP Asn-linked sugar chains [Hecker et al., Genes & Development 6:1213-1228 (1992)] and multiple conformers of PrP^{Sc} [Prusiner, S.B., Science 252:1515-1522 (1991)]. The patterns of PrP^{Sc} in Tg(MHu2M) mice were remarkably similar for the three inocula from humans dying of CJD.

The patterns of PrP^{Sc} accumulation in the brains of inoculated Tg(MHu2M) mice were markedly different for RML prions and Hu prions. However, RML prion inocula containing MoPrP^{Sc} stimulated the formation of more MoPrP^{Sc} while Hu prion inocula containing HuPrP^{CJD} triggered production of MHu2MPrP^{Sc}. The distribution of neuropathological changes characterized by neuronal vacuolation and astrocytic gliosis is similar to the patterns of PrP^{Sc} accumulation in the brains of Tg(MHu2M) mice inoculated with RML prions or Hu prions.

New approaches to investigating human prion diseases

The remarkable sensitivity of Tg(MHu2M) mice to Hu prions represents an important advance in neurodegenerative disease research. Based on the present disclosure regarding chimeric Hu/Mo PrP transgenes we conceived of a similar approach to the construction of Tg mice susceptible to BSE and scrapie sheep prions. Such

would be useful in detecting prion diseases in domestic animals. The importance of animal prion diseases is illustrated by BSE or "mad cow disease" in Great Britain, where >150,000 cattle have died. This prion disease BSE
5 is thought to have originated with cattle consuming meat and bone meal produced from sheep offal containing scrapie prions [Wilesmith, J.W., Semin. Viro. 2, 239-245].

The BSE epidemic has led to considerable concern
10 about the safety for humans of European beef and other cattle products. Epidemiologic studies over the past two decades have provided much data arguing that humans are unlikely to contract CJD from scrapie-infected sheep products [Harries-Jones et al., J. Neurol. Neurosurg.
15 Psychiatry 51:1113-1119 (1988); Cousens et al., J. Neurol. Neurosurg. Psychiatry 53:459-465 (1990); Brown et al., Neurology 37:895-904 (1987)]. There are seven amino acid substitutions which distinguish bovine from sheep PrP which must be considered in drawing conclusions
20 from sheep scrapie about the risk factors to humans from BSE. Whether any of these seven amino acid substitutions render bovine prions permissive in humans remains to be established. It may be that Tg(MHu2M) mice are susceptible to bovine as well as sheep prions. Of
25 perhaps even greater importance, Tg(MHu2M) mice have immediate application in the testing of pharmaceuticals for human prion contamination. The (Hu2M) mice described here provide a sensitive, reliable and economical bioassay for detecting the presence of human
30 prions.

Chimeric PrP Gene

Since the fundamental event underlying prion propagation seems to be a conformational change in PrP
35 [Pan et al., Proc. Natl. Acad. Sci. USA 90:10962-10966 (1993)] and mouse PrP differs from human PrP at 31 positions out of 254 [Kretzschmar et al., DNA 5:315-324

(1986)], we constructed modified PrP transgenes. Chimeric SHa/Mo transgenes have produced prions with new properties, the most useful being the chimeric SHa/Mo transgene labeled MH2M which carries 5 amino acid
5 substitutions found in SHaPrP lying between codons 94 and 188. [Scott et al., Cell 73:979-988 (1993)]. We made an analogous chimeric human/mouse PrP gene, which we call MHu2M, in which the same region of the mouse gene is replaced by the corresponding human sequence which
10 differs from mouse PrP at 9 codons as is shown in Figure 2.

We have found that mice expressing the MHu2M chimeric transgene are susceptible to human prions after abbreviated incubation times. More specifically, the
15 transgenic mice of the present invention which include the chimeric MHu2M gene will, after inoculation with human prions, develop disease symptoms attributed to the prions within about 200 days \pm 50 days. Further, 80% or more the transgenic mice of the invention inoculated with
20 human prions will develop symptoms of the disease, more preferably 98% or more of the mice will develop symptoms of the disease. According to experiments carried out, 100% of the transgenic MHu2M mice inoculated with human prions actually developed symptoms of the disease in
25 about 200 days \pm 50 days.

These findings indicate that murine cells cannot readily convert HuPrP^C into HuPrP^{Sc} but they can process MHu2MPrP^C into MHu2MPrP^{Sc}. Since Tg(MHu2M) mice develop neurodegeneration more rapidly than monkeys, they provide
30 a preferred host for bioassays of infectivity in tissues of humans dying of prion diseases. The Tg(MHu2M) mice disclosed herein provide an excellent system for assessing the sterility of pharmaceuticals prepared from human sources. Other transgenic mice which include the
35 prion protein gene of the animal in danger of infection can be used to test samples for prion diseases which can infect domestic animals such as sheep and cattle.

Chimeric MHu2M gene

Figure 1 is shown regarding how to create the chimeric MHu2M gene. At first, we engineered a new KpnI site in the HuPrP ORF cassette (shown shaded), changing nucleotide 282 from a cytosine to a thymine residue by PCR-mediated mutagenesis. This mutagenic change conserves the amino acid sequence of HuPrP. A second oligonucleotide primer complimentary to DNA sequences around the BstEII-cut product was used to replace the corresponding MoPrP gene fragment (the MoPrP gene is unshaded) creating the hybrid gene MHu2M. Microinjection of a cosSHa.Tet construct bearing this expression cassette resulted in founder animal Tg(MHu2M)FVB-B5378.

An expanded representation of the region of MHu2MPrP between codons 94 and 188 which is flanked by MoPrP sequences (Figure 2). MHu2MPrP differs from MoPrP by nine amino acids in the region between amino acids 96 and 167. These amino acid residues which are derived from HuPrP are shown on the lower section of the diagram; the amino acids at the same position of MoPrP are shown above. The discrepancy of amino acid positions is due to MoPrP having one less amino acid than HuPrP in the region immediately upstream from the replacement.

Artificial PrP Genes

The real power of the present invention lies in the understanding that a variety of different artificial PrP genes can be created which, when inserted into a first mammal, will render that mammal susceptible to infection with prions which normally only infect a second and genetically diverse mammal. There are nearly an infinite number of possible artificial PrP genes which would meet the basic criteria of the invention, i.e. rendering a mammal such as a mouse susceptible to infection with prions which normally infect only a genetically diverse mammal such as a human. The MHu2M gene of the invention is only one specific example of an artificial gene which

achieves the primary object of the invention. By reviewing Figures 3, 4 and 5 numerous other artificial gene possibilities will be deduced by those skilled in the art. Specifically, referring to Figure 3 one can readily determine the amino acid sequence of mouse PrP and observe the positions wherein the mouse PrP sequence differs with a human PrP sequence. Thus, to create an artificial gene one can substitute a codon (or sequence of codons) of a mouse PrP gene with a codon (or sequence of codons) of a human PrP gene at the same position which will encode a different amino acid - any (but not all) of the codons where different sequences appear can be used for substitution. Referring to Figure 4 it can be seen how it would be possible to produce artificial PrP genes wherein the resulting gene could be inserted into a mouse in order to render the mouse susceptible to infection with bovine prions. A similar strategy with respect to producing a mouse which would be susceptible to infection with sheep prions can be deduced from reviewing Figure 5. In addition to these possibilities those skilled in the art will recognize that, in certain instances, completely artificial nucleotide sequences can be used as corresponding substitutes for a portion of the natural sequence in order to obtain a useful artificial gene which, when inserted into an animal, will render that animal susceptible to infection with prions which normally would infect only a genetically diverse mammal.

Evidence of Disease

PrP^{sc} has been found in the brains of affected Tg(MHu2M) mice after inoculation with Hu(CJD) or Mo(RML) prions. Brain homogenates of Tg(MHu2M) were either left undigested or digested with proteinase K (BMB) at a final concentration of 20 µg/ml for 1 hour at 37°C (even numbered lanes). Samples were resolved by SDS-PAGE and then analyzed by Western blot.

The distribution of PrP^c and PrP^{sc} in clinically sick Tg(MHu2M) mice inoculated with Mo(RML) and Hu(CJD) prions were detected by the histoblot method. The histoblots included those of coronal sections through the region of the hippocampus and thalamus. Differences are observed between: (A) PrP^c in Mo(RML) infected mouse; (B) PrP^c in sporadic CJD RG-infected mouse; (C) PrP^{sc} in Mo(RML) infected mouse; (D) PrP^{sc} in sporadic CJD RG-infected mouse; (E) PrP^{sc} in sporadic CJD EC-infected mouse; and (F) PrP^{sc} in iatrogenic CJD (#364)-infected mouse.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the chimeric genes, transgenic mice and assays of the present invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1

Construction of Chimeric gene (MHu2M)

The source of the HuPrP ORF for construction of an expression cassette has been described [Hsiao et al., Nature 338:342-345 (1989)]. The construction of the MHu2M gene is described in connection with the description of Figure 1. All PrP ORF cassettes were flanked by Sall and XhoI, which cleave immediately adjacent to the PrP initiation and termination codons of the PrP ORF respectively, allowing for convenient subcloning into the ccs.SHaTet cosmid expression vector

[Scott et al., Cell 73:979-988 (1993)]. The isolation and screening of recombinant cosmid clones was achieved by methods which have been previously described [Scott et al., Cell 73:979-988 (1993)].

5

Example 2

Producing Transgenic Mice/Tg(MHu2M)

The nucleotide sequences of the HuPrP and MHu2MPrP ORFs of Example 1 were verified. The cosmid NotI
10 fragments, recovered from large-scale DNA preparations, were used for microinjection into the pronuclei of fertilized C57BL/6 X SJL or FVB/N oocytes as previously described [Scott et al., Cell 59:847-857 (1989); Scott et al., Protein Sci. 1:986-997 (1992)]. Genomic DNA
15 isolated from tail tissue of weaning animals was screened for the presence of incorporated transgenes using a probe that hybridizes to the 3'-untranslated region of the SHaPrP gene contained in the cosSHa.Tet vector [Scott et al., Protein Sci. 1:986-997 (1992)]. The offspring
20 obtained were tested and it was confirmed that the chimeric MHu2M gene was integrated into the genome of these offspring. As shown in Example 5 below, these mice were found to be susceptible to infection with human prions 100% of the time.

25

Example 3

Preparation of brain homogenates

A 10% [w/v] homogenate of a sample of thawed human
30 brain tissue was prepared in phosphate buffered saline lacking calcium and magnesium ions. The tissue was initially dissociated using a sterile disposable homogenizer, and this suspension was subjected to repeated extrusion through an 18 gauge syringe needle
35 followed by a 22 gauge needle. Samples for inoculation into test animals were diluted 10-fold. Homogenates of clinically sick Tg and non-Tg mouse brains were prepared

in the same way except for the omission of the initial dissociation step.

Example 4

5 Sources of prion inocula

Human inocula were derived from frozen brain tissues of patients in which the clinical diagnosis of CJD or GSS had been confirmed by histopathological examination of brain tissues and, in most cases, by prion protein analysis. In some cases, the PrP gene was amplified by PCR of DNA isolated from patient blood and the PrP sequence determined by DNA sequence analysis. No HuPrP mutations were detected in cases of sporadic or iatrogenic CJD. The RML isolate was obtained from Swiss mice [Chandler, R.L., Lancet 1:1378-1379 (1961)] from a closed colony at the Rocky Mountain Laboratory or in Swiss CD-1 mice obtained from Charles River Laboratories.

Example 5

20 Determination of Scrapie Incubation Periods

Transgenic mice as per Example 2 were inoculated intracerebrally with 30 μ l of brain extract using a 27 gauge needle inserted into the right parietal lobe. The preparation of inocula and criteria for diagnosis of scrapie in mice have been described [Carlson et al., Cell 46:503-511 (1986)]. Beginning 50 days after inoculation, the mice were examined for neurologic dysfunction every 3 days. When clinical signs of scrapie appeared, the mice were examined daily. When some animals whose death was obviously imminent were identified, their brains were taken for histopathologic studies (as per the procedures of Example 3) and confirmation of the diagnosis of scrapie.

Example 6Immunoblot Analysis

For the determination of the relative levels of PrP expression in Tg mouse and human brains, protein concentrations were determined by bicinchoninic acid assay and immuno dots blots as previously described [Scott et al., Cell 73:979-988 (1993)]. Samples for Western blot analysis were prepared and western blots were performed as described previously [Towbin et al., Proc. Natl. Acad. Sci. USA 76:4350-4354 (1979)], except that an enhanced chemiluminescent (ECL) detection method (Amersham) was used. The blot was exposed to X-ray film for 5-60 seconds. α -PrP RO73 rabbit antiserum was used at a final dilution of 1:5000 and 3F4 monoclonal antibody was also employed [Serban et al., Neurology 40:110-117 (1990)].

Example 7Tg(MHu2MPrP) Mice Are Susceptible to Human Prions

Using information from a systematic study of chimeric SHa/Mo PrP genes, a chimeric Hu/Mo PrP gene analogous to MH2M was constructed as per Example 1. This gene, which we call MHu2M, differs from MoPrP at 9 positions, all of which lie between codons 94 and 188 as is shown in Figure 2. A Tg mouse was constructed expressing the MHu2M PrP gene and the founder designated Tg(MHu2M)FVB-B5378 as per Example 2. By serial dilution of Tg(MHu2M) mouse and human brain homogenates, we estimate that the level of MHu2MPrP^C in the brains of these Tg(MHu2M)FVB-B5378 mice are similar to those of HuPrP^C found in human brain using an immuno dot blotting procedure.

Tg(MHu2M) mice as per Example 2 were inoculated with brain homogenates from three unrelated Caucasian patients who had died of CJD. Two of the three patients died of sporadic CJD: one (R.G.) was a 56-year-old American female; the other (E.C.) was a 61-year-old Canadian

female. In both cases, cerebrocortical biopsy showed severe spongiform degeneration. The third (#364) was a British youth who had contracted iatrogenic CJD after treatment for hypopituitarism with human growth hormone (HGH) derived from cadaveric pituitaries [Collinge et al., Lancet 337:1441-1442 (1991)]. Brain homogenates from all three CJD patients exhibited protease-resistant PrP by Western immunoblotting. This protease-resistant isoform of PrP is designated PrP^{Sc}, or often PrP^{CJD} when it is found in humans.

All of the Tg(MHu2M) mice inoculated with homogenates from the CJD patients described above developed signs of central nervous system (CNS) dysfunction ~200 days after inoculation (see Table 1 below). The clinical signs were similar to those of murine scrapie. After developing clinical signs, the inoculated Tg(MHu2M) mice died rapidly, often within a day. Two of the eight uninoculated Tg(MHu2M) mice are now >500 days of age and remain well; the youngest of the other six uninoculated animals is older than the age at which inoculated Tg(MHu2M) mice developed signs of CNS dysfunction.

Inoculation of Tg(MHu2M) mice with Mo(RML) prions passaged in mice produced disease in 178 ± 3 days, which is ~40 longer than Mo(RML) prions in non-Tg mice. Prolongation of incubation times in mice expressing non-murine transgenes is well established, and occurs presumably because the foreign PrP^C molecule inhibits the conversion of MoPrP^C into MoPrP^{Sc} [Prusiner et al., Cell 63:673-686 (1990)]. In contrast to Tg(MHu2M) mice, incubation times for RML prions in Tg(MH2M) mice were the same as those of the non-Tg mice [Scott et al., Cell 73:979-988 (1993)].

Table 1. Incubation of human (CJD) and mouse (RML) prion inocula in Tg(MHu2M)FVB-B5378 mice

Source	Inoculum	Incubation Times (mean days \pm SE)		Illness	Death ^b
		No. ^a	Range (days)		
Sporadic CJD	RG	8/8	225-249	238 \pm 3.2	240 \pm 5.4(3)
Sporadic CJD	EC	7/7	202-229	218 \pm 4.6	N.D.
Latrogenic CJD	#364	9/9	221-245	232 \pm 2.9	235 \pm 3.9(5)
Mo	RML	19/19	155-195	178 \pm 3.3	203 \pm 2.0(14)

Number of animals developing clinical sickness (neurological dysfunction) divided by the total number of animals inoculated. In the case of inoculum RG, three animals were found dead after 224, 238, and 243 days before a clinical diagnosis could be made. In the case of inoculum EC, two animals were found dead after 225 and 226 days before a clinical diagnosis could be made. In each case, these animals died at the same time that clinical diagnosis was made in other inoculated animals. The number of mice dying of scrapie is shown in parentheses. Mice sacrificed for pathological examination are excluded from these calculations.

Example 8

Comparative Example

Tg(HuPrP) Mice Are Resistant to Human Prions

Tg mice expressing HuPrP were produced using the HuPrP gene ORF, which had been cloned into the cosSHa.Tet expression vector [Scott et al., Protein Sci. 1:986-997 (1992)]. Microinjection of outbred C57B6/SJL and inbred FVB mouse embryos resulted in two founder transgenic animals designated Tg(HuPrP)B6SJL-110 and Tg(HuPrP)FVB-152. We estimated by serial dilution of brain homogenates and immuno dot blotting, that the level of PrP^C in the brains of the progeny of these founders

express HuPrP at levels 4- to 8-fold higher than the level of HuPrP found in the human brain.

To determine whether expression of HuPrP in Tg(HuPrP)B6SJL-110 and Tg(HuPrP)FVB-152 conferred susceptibility to human prions, incubation periods were measured after inoculation of Tg(HuPrP) and non-Tg mice with brain extracts from 18 patients that had died of sporadic CJD, iatrogenic CJD, familial CJD or GSS. From experiments performed over the past 2.5 years, we concluded that the two lines of Tg(HuPrP) mice were no more responsive than non-Tg mice to human prions (see Table 2 below). The rate of transmission to Tg(HuPrP) mice was 8.3% (14 clinically sick mice out of 169 mice) which was similar to a transmission rate of 10.3% in control non-Tg mice (6 clinically sick mice out of 58 mice). In the infrequent event of a positive transmission, incubation times were extremely long ranging, from 590 days to 840 days in both Tg(HuPrP) and non-Tg mice. By this late time, many animals had died of intercurrent illnesses which complicated diagnosis. The difficulty of interpreting transmissions occurring after extremely long incubation periods is compounded by the heightened potential for artifactual results due to low levels of contaminating prions.

Statistical analysis shows that the frequency of Hu prion transmission to Tg(MHu2MPrP) mice compared to Tg(HuPrP) and non-Tg mice is highly significant using the Fisher's exact test, $p < 10^{-7}$ [Mehta et al., J. Am. Stat. Assn. 78:(392) 427-434 (1983)]. When Hu prion transmission to Tg(HuPrP) mice was compared to non-Tg mice, the frequencies were similar, $p = 0.79$.

To confirm the clinical diagnosis of prion disease, 5 ill Tg(HuPrP) and 1 non-Tg mice were sacrificed and brain extracts were examined for the presence of PrP^{Sc} by Western blotting with the α -PrP antibodies, 3F4 mAb and RO73 antiserum [Kascsak et al., J. Virol. 61:3688-3693 (1987); Serban et al., Neurology 40:110-117 (1990)]. The

3F4 mAb reacts specifically with HuPrP allowing discrimination from MoPrP. MoPrP^s was detected in the brain of the non-Tg mouse inoculated with sporadic CJD inoculum #87011 which developed clinical signs after 756 days, while 3F4-reactive PrP^{Sc} was detected in the brains of two Tg(HuPrP) mice which developed clinical signs after 589 days post-inoculation with iatrogenic CJD inoculum #170. Notable is the finding that the protease-resistant fragments of HuPrP^{Sc} from ill Tg(HuPrP) mice migrate more rapidly on SDS-PAGE than do those of HuPrP^{CJD} from human CJD brain and MoPrP^{Sc} from non-Tg mouse brain. Whether or not this reflects more extensive proteolysis of the N-terminus due to differences in conformation that result from altered folding of HuPrP in a mouse cell remains to be established. The equivalent transmission rates of human prions in Tg(HuPrP) and non-Tg mice indicate that this is a rare event with the same frequency of occurrence as the stochastic conversion of MoPrP^C to MoPrP^{Sc} induced by human prions.

The absence of either RO73- or 3F4-reactive PrP^{Sc} in the brains of 3 out of the 6 mice analyzed may reflect the difficulty of accurately diagnosing prion disease in elderly animals. Some of the mice inherited prion diseases of both humans and Tg mice exhibit little or undetectable levels of protease-resistant PrP; yet, based on transmission studies, their brains contain prions and they show clear spongiform degeneration (Medori, R. et al, N. Engl. J. Med. 326, 444-449 (1992); Hsiao et al., (In preparation) (1994)).

In contrast to Tg(MHu2M) mice, Hu prions from patient RG have not transmitted to either Tg(HuPrP) or non-Tg mice after >330 days (see Table 2 below). Attempts to transmit preparations enriched for Hu prion rods prepared from the brain of patient RG have likewise been negative for >300 days. In addition, inoculum from the iatrogenic CJD case (#364) has produced illness in

neither Tg(HuPrP) nor non-Tg mice after >780 days (as shown in Table 2 below).

Table 2. Incubation times in Tg(HuPrP)FVB-152 and Tg(HuPrP)B6SJL-110 mice after inoculation with brain extracts from patients with human prion diseases				
	Host	Inoculum	(n/n) ^a	Incubation times (days ± SE) ^b
5	Tg152	Sporadic CJD(#87011)	1/10	706
	Non-Tg	Sporadic CJD(#87011)	3/5	697.3 ± 51
10	Tg 152	Sporadic CJD(#88037)	3/10	680 ± 28
	Tg 152	Sporadic CJD(RG)	0/10	
15	Non-Tg	Sporadic CJD(RG)	0/10	
	Tg 152	Sporadic (RG) Rods	0/8	
20	Non-Tg	Sporadic (RG) Rods	0/8	
	Tg 152	Codon 102 GSS(#87027)	4/10	724 ± 16
25	Non-Tg	Codon 102 GSS(#87027)	0/10	679
	Tg 152	Codon 102 GSS(#87031)	0/10	
30	Non-Tg	Codon 102 GSS(#87031)	1/5	742
	Tg 152	Codon 178 F-CJD	0/8	
35	Non-Tg	Codon 178 F-CJD	0/8	
	Tg 110	Sporadic CJD(#87036)	0/8	
30	Non-Tg	Sporadic CJD(#87036)	1/5	838
	Tg 110	Iatrogenic CJD(#703)	0/10	
35	Non-Tg	Iatrogenic CJD(#703)	0/5	
	Tg 110	Iatrogenic CJD(#170)	2/10	589 ± 0
	Non-Tg	Iatrogenic CJD(#170)	0/5	

5	Tg 110	Iatrogenic CJD(#364)	0/10	
	Non-Tg	Iatrogenic CJD(#364)	0/5	
	Tg 110	Codon 200 F-CJD	1/8	791
	Tg 110	Codon 217 GSS	1/8	874
	Tg 110	Codon 102 GSS-A	0/10	
10	Tg 110	Codon 102 GSS-B	1/8	694
	Tg 110	Codon 117 GSS	0/8	

15 * Number of animals developing clinical sickness divided by the total number of animals inoculated.

^b Refers to time to diagnosis of illness.

^{c-f} Patients from which inoculum were derived are described in the following publications: [Collinge et al., Lancet 337:1441-1442 (1991); Hsiao et al., Nature 338:342-345 (1989); Hsiao et al., Neurology 41:681-684 (1991)].

Example 9

Formation of MHu2MPrP^{Sc} or MoPrP^{Sc}

In the Brains of Tg(MHu2M) Mice

25 Some clinically sick Tg(MHu2M) mice inoculated with each of the three CJD prion inocula or RML prions were sacrificed for histopathological verification of disease and for prion protein analysis. Western blots of brain
30 homogenates from Tg(MHu2M) mice infected with Hu prions probed with RO73 and 3F4 α -PrP antibodies revealed the presence of protease-resistant PrP^{Sc} which reacted with the 3F4 monoclonal antibody showing this protease-resistant product to be MHu2M PrP^{Sc}. The epitope
35 recognized by this antibody consists of a pair of methionine residues at positions 109 and 112 in PrP [Rogers et al., J. Immunol. 147:3568-3574 (1991)] which

are contained in MHu2M but not in MoPrP as can be seen by the mouse/human comparison of figure 2. The polyclonal rabbit α -PrP antiserum R073 diluted 1:5000 was poorly reactive with MHu2MPrP^{Sc} as well as HuPrP^C and HuPrP^{Sc} from diseased human brains. Brain homogenates from Tg(MHu2M) mice infected with RML prions contained PrP^{Sc} which was detectable only with R073 and not 3F4 α -PrP antibodies, indicating that Tg(MHu2M) mice are capable of producing MoPrP^{Sc} but not MHu2MPrP^{Sc} in response to RML prions previously passaged in mice. While these findings are similar to those reported for Tg(SHaPrP) mice [Scott et al., Cell 59:847-857 (1989)], they contrast with those found for Tg(MH2MPrP) mice where MH2MPrP^{Sc} was formed in response to RML prions [Scott et al., Cell 73:979-988 (1993)].

Example 10

Regional Distribution of PrP^{Sc} and Patterns of Neuropathology

The distribution of Mo and MHu2M PrP^C and PrP^{Sc} is shown in histoblots of coronal brain sections through the hippocampus and thalamus of Tg(MHu2M) mice inoculated with RML or CJD prions. The weak immunoreactivity of MHu2M PrP with R073 permitted a degree of analysis which had not been previously possible in Tg mice expressing SHaPrP or MH2MPrP because these PrP species react with this antibody. The pattern of PrP^{Sc} deposition was highly dependent upon the species of origin of the infectious prions. When inoculated with RML prions, histoblots of the brains of Tg(MHu2M) were similar to those of CD-1 mice infected with RML prions, revealing a diffuse pattern of MoPrP^{Sc} deposition in the hippocampus, thalamus, hypothalamus and all layers of the neocortex. The histoblot pattern of was strikingly different for Tg(MHu2M) mice inoculated with CJD prions. The deposition of MHu2MPrP^{Sc} was confined primarily to the deep layers of the neocortex, the thalamus, particularly

the ventral posterior medial thalamic nucleus, the hypothalamus and the putamen. The hippocampal region and the outer layers of the neocortex were spared. Interestingly, while the hippocampus was completely devoid of MHu2MPrP^{sc}, this region showed the most intense MHu2MPrP^{sc} signal. The same pattern of MHu2MPrP^{sc} deposition was consistently observed in histoblots of Tg(MHu2M) mice inoculated with all three CJD prion isolates prepared from human brain. It is noteworthy that the pattern of MHu2MPrP^{sc} deposition is similar to the pattern of PrP^{CJD} deposition observed in histoblots of the brain from which inoculum RG was derived [Taraboulos et al., Proc. Natl. Acad. Sci. USA 89:7620-7624 (1992)]. The spongiform degeneration in the brains of Tg(MHu2M) mice infected with Mo(RML) and Hu(CJD) prions reflected the patterns of PrP^{sc} accumulation described above.

The instant invention is shown and described herein in what is considered to be a most practical and preferred embodiments. It is recognized, however, that departures may be made from which are within the scope of the invention and that modifications will occur to one who is skilled in the art upon reading this disclosure.

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: The Regents of the University of California
- (ii) TITLE OF INVENTION: METHOD OF DETECTING PRIONS IN A SAMPLE AND TRANSGENIC ANIMAL USED FOR SAME
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Robbins, Silver & Carson
 (B) STREET: 201 N. Figueroa Street, 5th Floor
 (C) CITY: Los Angeles
 (D) STATE: CA
 (E) COUNTRY: USA
 (F) ZIP: 90012-2628
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
 (B) FILING DATE:
 (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Berliner, Robert
 (B) REGISTRATION NUMBER: 20,121
 (C) REFERENCE/DOCKET NUMBER: 5555-313
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: (213) 977-1001
 (B) TELEFAX: (213) 977-1003

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 254 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: MOUSE PRION PROTEIN, MoPrP
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- ```

Met Ala Asn Leu Gly Tyr Trp Leu Leu Ala Leu Phe Val Thr Met Trp
1 5 10 15

Thr Asp Val Gly Leu Cys Lys Lys Arg Pro Lys Pro Gly Gly Trp Asn
20 25 30

Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro Gly Gly Asn Arg
35 40 45

Tyr Pro Pro Gln Gly Gly Thr Trp Gly Gln Pro His Gly Gly Gly Trp
50 55 60

Gly Gln Pro His Gly Gly Ser Trp Gly Gln Pro His Gly Gly Ser Trp
65 70 75 80

Gly Gln Pro His Gly Gly Gly Trp Gly Gln Gly Gly Thr His Asn
85 90 95

```

42

Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn Leu Lys His Val Ala  
 100 105 110  
 Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly Leu Gly Gly Tyr Met  
 115 120 125  
 Leu Gly Ser Ala Met Ser Arg Pro Met Ile His Phe Gly Asn Asp Trp  
 130 135 140  
 Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr Arg Tyr Pro Asn Gln Val  
 145 150 155 160  
 Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn Gln Asn Asn Phe Val His  
 165 170 175  
 Asp Cys Val Asn Ile Thr Ile Lys Gln His Thr Val Thr Thr Thr Thr  
 180 185 190  
 Lys Gly Glu Asn Phe Thr Glu Thr Asp Val Lys Met Met Glu Arg Val  
 195 200 205  
 Val Glu Gln Met Cys Val Thr Gln Tyr Gln Lys Glu Ser Gln Ala Tyr  
 210 215 220  
 Tyr Asp Gly Arg Arg Ser Ser Ser Thr Val Leu Phe Ser Ser Pro Pro  
 225 230 235 240  
 Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu Ile Val Gly  
 245 250

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 253 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: HUMAN PRION PROTEIN, HuPrP

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Asn Leu Gly Cys Trp Met Leu Val Leu Phe Val Ala Thr Trp  
 1 5 10 15  
 Ser Asp Leu Gly Leu Cys Lys Lys Arg Pro Lys Pro Gly Gly Trp Asn  
 20 25 30  
 Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro Gly Gly Asn Arg  
 35 40 45  
 Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly  
 50 55 60  
 Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly Gly Gly  
 65 70 75 80  
 Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Gly Gly Gly Thr His  
 85 90 95  
 Ser Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn Met Lys His Met  
 100 105 110  
 Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly Leu Gly Gly Tyr  
 115 120 125

42

Met Leu Gly Ser Ala Met Ser Arg Pro Ile Ile His Phe Gly Ser Asp  
 130 135 140

Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met His Arg Tyr Pro Asn Gln  
 145 150 155 160

Val Tyr Tyr Arg Pro Met Asp Glu Tyr Ser Asn Gln Asn Asn Phe Val  
 165 170 175

His Asp Cys Val Asn Ile Thr Ile Lys Gln His Thr Val Thr Thr Thr  
 180 185 190

Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Val Lys Met Met Glu Arg  
 195 200 205

Val Val Glu Gln Met Cys Ile Thr Gln Tyr Glu Arg Glu Ser Gln Ala  
 210 215 220

Tyr Tyr Gln Arg Gly Ser Ser Met Val Leu Phe Ser Ser Pro Pro Val  
 225 230 235 240

Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu Ile Val Gly  
 245 250

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 263 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: BOVINE PRION PROTEIN, B<sub>ov</sub>PrP

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Val Lys Ser His Ile Gly Ser Trp Ile Leu Val Leu Phe Val Ala  
 1 5 10 15

Met Trp Ser Asp Val Gly Leu Cys Lys Lys Arg Pro Lys Pro Gly Gly  
 20 25 30

Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro Gly Gly  
 35 40 45

Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly Gln Pro His Gly  
 50 55 60

Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly  
 65 70 75 80

Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly  
 85 90 95

Gly Gly Gly Trp Gly Gln Gly Gly Thr His Gly Gln Trp Asn Lys Pro  
 100 105 110

Ser Lys Pro Lys Thr Asn Met Lys His Val Ala Gly Ala Ala Ala Ala  
 115 120 125

Gly Ala Val Val Gly Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala Met  
 130 135 140

Ser Arg Pro Leu Ile His Phe Gly Ser Asp Tyr Glu Asp Arg Tyr Tyr  
 145 150 155 160

43

Arg Glu Asn Met His Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val  
 165 170 175  
 Asp Gln Tyr Ser Asn Gln Asn Asn Phe Val His Asp Cys Val Asn Ile  
 180 185 190  
 Thr Val Lys Glu His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn Phe  
 195 200 205  
 Thr Glu Thr Asp Ile Lys Met Met Glu Arg Val Val Glu Gln Met Cys  
 210 215 220  
 Val Thr Gln Tyr Gln Lys Glu Ser Gln Ala Tyr Tyr Asp Gln Gly Ala  
 225 230 235 240  
 Ser Val Ile Leu Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe  
 245 250 255  
 Leu Ile Phe Leu Ile Val Gly  
 260

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 255 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: SHEEP PRION PROTEIN, SHPrP

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Val Lys Ser His Ile Gly Ser Trp Ile Leu Val Leu Phe Val Ala  
 1 5 10 15  
 Met Trp Ser Asp Val Gly Leu Cys Lys Lys Arg Pro Lys Pro Gly Gly  
 20 25 30  
 Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro Gly Gly  
 35 40 45  
 Asn Arg Tyr Pro Pro Gln Gly Gly Gly Gly Trp Gly Gln Pro His Gly  
 50 55 60  
 Gly Gly Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Pro His Gly  
 65 70 75 80  
 Gly Ser Trp Gly Gln Pro His Gly Gly Gly Gly Trp Gly Gln Gly Gly  
 85 90 95  
 Ser His Ser Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn Met Lys  
 100 105 110  
 His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly Leu Gly  
 115 120 125  
 Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Leu Ile His Phe Gly  
 130 135 140  
 Asn Asp Tyr Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr Arg Tyr Pro  
 145 150 155 160  
 Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn Gln Asn Asn  
 165 170 175

44

Phe Val His Asp Cys Val Asn Ile Thr Val Lys Gln His Thr Val Thr  
180 185 190  
Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Ile Lys Ile Met  
195 200 205  
Glu Arg Val Val Glu Gln Met Cys Ile Thr Gln Tyr Gln Arg Glu Ser  
210 215 220  
Gln Ala Tyr Tyr Gln Arg Gly Ala Ser Val Ile Leu Phe Ser Ser Pro  
225 230 235 240  
Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu Ile Val Gly  
245 250 255

CLAIMS

1. An artificial PrP gene characterized such that when inserted into the genome of a first mammal  
5 renders the mammal susceptible to infection with prions which normally infect only a genetically diverse second animal.

2. The artificial PrP gene of claim 1,  
10 wherein the first mammal is a mouse or a rat and the second genetically diverse mammal is selected from the group consisting of a human, a cow and a sheep.

3. The artificial PrP gene of claim 1,  
15 wherein the gene is comprised of a natural codon sequence of a PrP gene of the first mammal with one or more, but not all, of its codons replaced with a different codon of a natural PrP gene of the second genetically diverse animal.

20

4. A chimeric gene, comprising:  
C-terminus and N-terminus codon sequences of  
a PrP gene of a mammal of a first genus;  
codon sequences of a PrP gene of a mammal of  
25 a second genus operatively connected between the N-terminus and C-terminus codon sequences from the mammal of the first genus, wherein the chimeric gene renders a mammal of the first genus susceptible to infection with a prion normally infectious only to a mammal of the  
30 second genus when the chimeric gene is operably inserted into the genome of a mammal of the first genus.

5. The chimeric gene of claim 4, wherein the first genus is selected from the group consisting of Mus,  
35 Rattus, Oryctolagus, and Mesocricetus.

6. The chimeric gene of claim 4, wherein the second genus is selected from the group consisting of Bos, Ovis, Sus, and Homo.

5 7. The chimeric gene of claim 4, wherein the mammal of the first genus is a mouse and the mammal of the second genus is a human.

10 8. A transgenic mammal having a genome comprised of an artificial PrP gene wherein the mammal is susceptible to infection with a prion which does not normally infect a species of the mammal lacking the chimeric PrP gene.

15 9. The transgenic mammal of claim 8, belonging to a genus selected from the group consisting of Mus, Rattus, Oryctolagus, and Mesocricetus.

20 10. The transgenic mammal of claim 8, wherein the artificial PrP gene comprises a portion of a PrP gene from a different genus of mammal.

25 11. The transgenic mammal of claim 10, wherein the different genus of mammal is selected from the group consisting of Bos, Ovis, Sus, and Homo.

30 12. The transgenic mammal of claim 11, wherein the different genus of mammal is of the species Homo sapiens.

13. The transgenic mammal of claim 8, wherein the artificial gene is a mouse PrP gene having a portion thereof replaced with a human PrP gene.

35 14. The transgenic mammal of claim 13, wherein the artificial gene is MHu2M.

15. The transgenic mammal of claim 9, wherein the mammal has an 80% or greater chance of developing a disease as a result of being inoculated with a prion which does not normally infect a species of the mammal lacking the artificial PrP gene.

16. The transgenic mammal of claim 15, wherein the mammal has a 98% or greater chance of developing a disease as a result of inoculation with a prion which does not normally infect a species of the mammal lacking the artificial PrP gene.

17. The transgenic mammal of claim 8, wherein the mammal will exhibit a disease incubation time of about 300 days or less.

18. The transgenic mammal of claim 17, wherein the incubation time is about 200 days  $\pm$  50 days.

19. A method of testing a sample for the presence of prion, comprising:

inoculating a transgenic mammal with the sample, wherein the mammal has a genome comprised of an artificial PrP gene and is susceptible to infection with a prion which does not normally infect a species of a mammal lacking the artificial PrP gene; and

observing the transgenic mammal in order to determine if the mammal develops symptoms of prion infection.

20. The method of claim 19, wherein the transgenic mammal is a mouse.



21. The method of claim 19, wherein the artificial gene is a chimeric gene comprised of C-terminus and N-terminus codon sequences of the PrP gene of the mouse and, has fused operately thereto, codon  
5 sequences of a PrP gene of a human.

22. The method of claim 19, wherein the sample is a pharmaceutical formulation containing a therapeutically active component extracted from a human  
10 source.

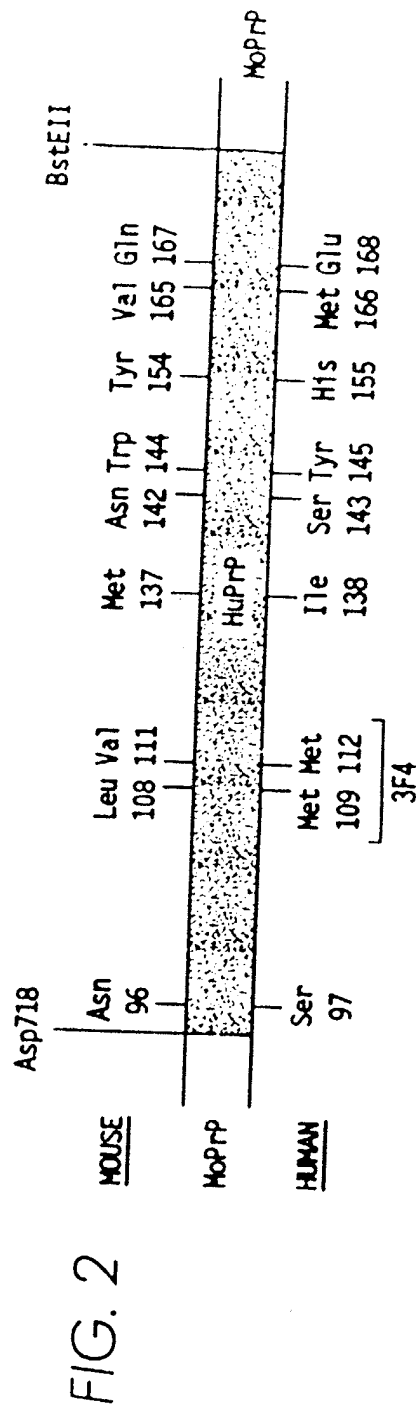
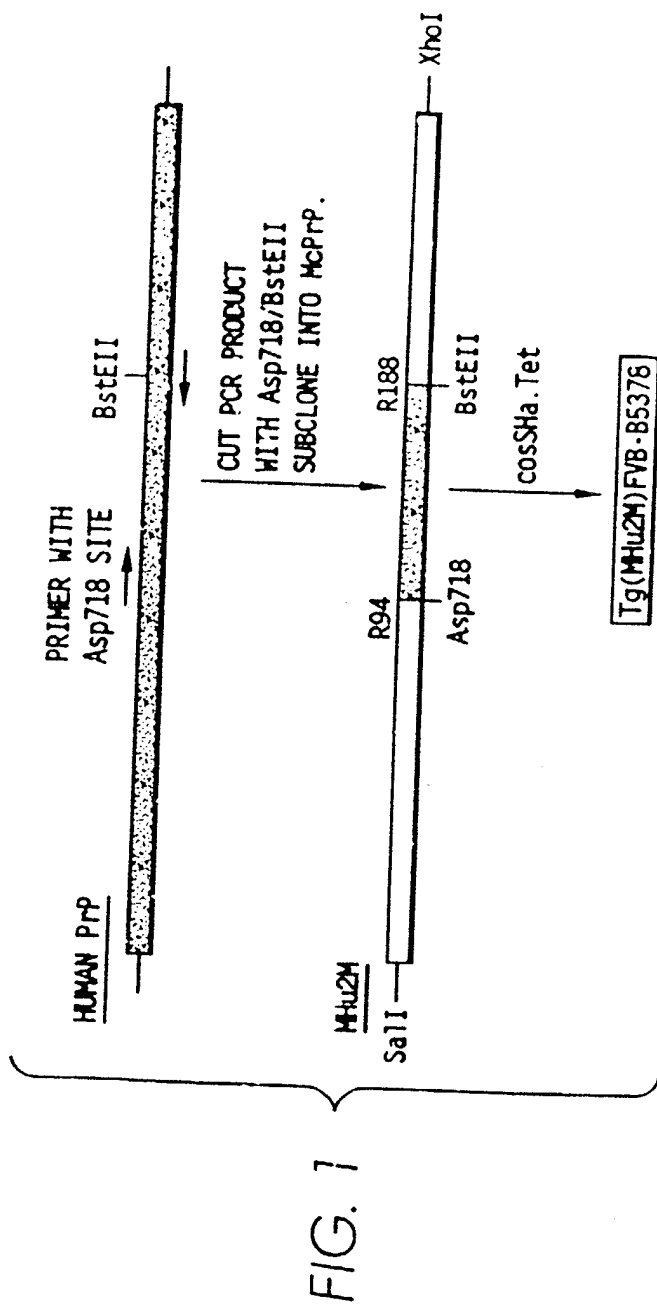
23. The method of claim 22, wherein the pharmaceutical formulation is a formulation selected from the group consisting of injectables, orals, creams,  
15 suppositories, and intrapulmonary delivery formulations.

24. The method of claim 19, wherein the sample is a cosmetic.

20 25. A method of determining the cause of death of an animal, comprising:  
extracting brain tissue from an animal that has died;

inoculating a transgenic mammal with  
25 extracted brain tissue wherein the transgenic mammal has a genome comprised of an artificial PrP gene and is susceptible to infection with a prion from the animal that has died, which prion does not normally infect a species of a mammal lacking the artificial PrP gene; and

30 observing the transgenic mammal in order to determine if the transgenic mammal develops symptoms of prion infections.



## FIG. 3

Mo Met Ala Asn Leu Gly Tyr Trp Leu Leu Ala Leu Phe Val Thr Met Trp 16  
Hu Cys Met Val Ala Thr

Mo Thr Asp Val Gly Leu Cys Lys Lys Arg Pro Lys Pro Gly Gly Trp Asn 32  
Hu Ser Leu

Mo Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro Gly Gly Asn Arg 48  
Hu

Mo Tyr Pro Pro Gln Gly Gly --- Thr Trp Gly Gln Pro His Gly Gly Gly 63  
Hu Gly Gly

Mo Trp Gly Gln Pro His Gly Gly Ser Trp Gly Gln Pro His Gly Gly Ser 79  
Hu Gly Gly

Mo Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln Gly Gly Gly Thr His 95  
Hu

Mo Asn Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn Leu Lys His Val 111  
Hu Ser Met Met

Mo Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly Leu Gly Gly Tyr 127  
Hu

Mo Met Leu Gly Ser Ala Met Ser Arg Pro Met Ile His Phe Gly Asn Asp 143  
Hu Ile Ser

Mo Trp Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr Arg Tyr Pro Asn Gln 159  
Hu Tyr His

Mo Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn Gln Asn Asn Phe Val 175  
Hu Met Glu

Mo His Asp Cys Val Asn Ile Thr Ile Lys Gln His Thr Val Thr Thr Thr 191  
Hu

Mo Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Val Lys Met Met Glu Arg 207  
Hu

Mo Val Val Glu Gln Met Cys Val Thr Gln Tyr Gln Lys Glu Ser Gln Ala 223  
Hu Ile Glu Arg

Mo Tyr Tyr Asp Gly Arg Arg Ser Ser Ser Thr Val Leu Phe Ser Ser Pro 239  
Hu Gln --- --- Gly Met

Mo Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu Ile Val Gly 254  
Hu

3/4

## FIG. 4

Mo Met Ala Asn Leu --- --- Gly Tyr Trp Leu Leu Ala Leu Phe Val Thr 14  
 Bo Val Lys Ser His Ile Ser Ile Val Ala

Mo Met Trp Thr Asp Val Gly Leu Cys Lys Lys Arg Pro Lys Pro Gly Gly 30  
 Bo Ser

Mo --- Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro Gly 45  
 Bo

Mo Gly Asn Arg Tyr Pro Pro Gln Gly Gly --- Thr Trp Gly Gln Pro His 60  
 Bo Gly Gly

Mo Gly Gly Gly Trp Gly Gln Pro His Gly Gly Ser Trp Gly Gln Pro His 76  
 Bo Gly

Mo Gly Gly Ser Trp Gly Gln Pro His Gly Gly Gly Trp Gly Gln --- --- 90  
 Bo Gly Pro His

Mo --- --- --- --- --- --- Gly Gly Gly Thr His Asn Gln Trp Asn Lys 100  
 Bo Gly Gly Gly Gly Trp Gly Gln Gly

Mo Pro Ser Lys Pro Lys Thr Asn Leu Lys His Val Ala Gly Ala Ala Ala 116  
 Bo Met

Mo Ala Gly Ala Val Val Gly Gly Leu Gly Gly Tyr Met Leu Gly Ser Ala 132  
 Bo

Mo Met Ser Arg Pro Met Ile His Phe Gly Asn Asp Trp Glu Asp Arg Tyr 148  
 Bo Leu Ser Tyr

Mo Tyr Arg Glu Asn Met Tyr Arg Tyr Pro Asn Gln Val Tyr Tyr Arg Pro 164  
 Bo His

Mo Val Asp Gln Tyr Ser Asn Gln Asn Asn Phe Val His Asp Cys Val Asn 180  
 Bo

Mo Ile Thr Ile Lys Gln His Thr Val Thr Thr Thr Thr Lys Gly Glu Asn 200  
 Bo Val Glu

Mo Phe Thr Glu Thr Asp Val Lys Met Met Glu Arg Val Val Glu Gln Met 212  
 Bo Ile

Mo Cys Val Thr Gln Tyr Gln Lys Glu Ser Gln Ala Tyr Tyr Asp Gly Arg 228  
 Bo Gln ---

Mo Arg Ser Ser Ser Thr Val Leu Phe Ser Ser Pro Pro Val Ile Leu Leu 244  
 Bo --- Gly Ala Val Ile

Mo Ile Ser Phe Leu Ile Phe leu Ile Val Gly 254  
 Bo

SUBSTITUTE SHEET (RULE 26)

## FIG. 5

Mo Met Ala Asn Leu --- --- Gly Tyr Trp Leu Leu Ala Leu Phe Val Thr 14  
 Sh Val Lys Ser His Ile Ser Ile Val Ala

Mo Met Trp Thr Asp Val Gly Leu Cys Lys Lys Arg Pro Lys Pro Gly Gly 30  
 Sh Ser

Mo --- Trp Asn Thr Gly Gly Ser Arg Tyr Pro Gly Gln Gly Ser Pro Gly 45  
 Sh

Mo Gly Asn Arg Tyr Pro Pro Gln Gly Gly --- Thr Trp Gly Gln Pro His 60  
 Sh Gly Gly

Mo Gly Gly Gly Trp Gly Gln Pro His Gly Gly Ser Trp Gly Gln Pro His 76  
 Sh Gly

Mo Gly Gly Ser Trp Gly Gln Pro His Gly Gly Gly --- Trp Gly Gln Gly 91  
 Sh Gly

Mo Gly Gly Thr His Asn Gln Trp Asn Lys Pro Ser Lys Pro Lys Thr Asn 107  
 Sh Ser --- His Ser

Mo Leu Lys His Val Ala Gly Ala Ala Ala Ala Gly Ala Val Val Gly Gly 123  
 Sh Met

Mo Leu Gly Gly Tyr Met Leu Gly Ser Ala Met Ser Arg Pro Met Ile His 139  
 Sh Leu

Mo Phe Gly Asn Asp Trp Glu Asp Arg Tyr Tyr Arg Glu Asn Met Tyr Arg 155  
 Sh Tyr

Mo Tyr Pro Asn Gln Val Tyr Tyr Arg Pro Val Asp Gln Tyr Ser Asn Gln 171  
 Sh

Mo Asn Asn Phe Val His Asp Cys Val Asn Ile Thr Ile Lys Gln His Thr 187  
 Sh Val

Mo Val Thr Thr Thr Thr Lys Gly Glu Asn Phe Thr Glu Thr Asp Val Lys 203  
 Sh Ile

Mo Met Met Glu Arg Val Val Glu Gln Met Cys Val Thr Gln Tyr Gln Lys 219  
 Sh Ile Ile Arg

Mo Glu Ser Gln Ala Tyr Tyr Asp Gly Arg Arg Ser Ser Ser Thr Val Leu 235  
 Sh Gln --- --- Gly Ala Val Ile

Mo Phe Ser Ser Pro Pro Val Ile Leu Leu Ile Ser Phe Leu Ile Phe Leu 251  
 Sh

Mo Ile Val Gly 254  
 Sh

## INTERNATIONAL SEARCH REPORT

Int. national application No.

PCT/US95/04426

| <b>A. CLASSIFICATION OF SUBJECT MATTER</b><br>IPC(6) C07H 15/12, C12N 15/00; A61K 49/00<br>US CL 536/23.4, 800/2, 424/9<br>According to International Patent Classification (IPC) or to both national classification and IPC                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                         |                                                                                                                                                                                                                                               |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------|-----|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|-----|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|-----|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|-------------------------------------------|------------------------------------------------------------------------------|--|--|--------------------------------------------------------------------------------------------------------|--|--|
| <b>B. FIELDS SEARCHED</b><br>Minimum documentation searched (classification system followed by classification symbols)<br>U.S. : 536/23.4; 800/2, 424/9<br>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched<br>Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)<br>APS, CHEMICAL ABSTRACTS                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                                                                                                                                         |                                                                                                                                                                                                                                               |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| <b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                                                                                                                                                                                         |                                                                                                                                                                                                                                               |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| Category*                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                      | Relevant to claim No.                                                                                                                                                                                                                         |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| X                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | US, A, 5,237,056 (FISCHBACH) 17 August 1993, see entire document.                                                                                                                       | 1-7                                                                                                                                                                                                                                           |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| X                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | Cell, Volume 73, 04 June 1993, M. Scott et al, "Propagation of Prions with Artificial Properties in Transgenic Mice Expressing Chimeric PrP Genes", pages 979-988, see entire document. | 8-25                                                                                                                                                                                                                                          |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| <input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                                                                                         |                                                                                                                                                                                                                                               |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| <table border="0"> <tr> <td>* Special categories of cited documents:</td> <td>"T"</td> <td>later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principles or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X"</td> <td>documents of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E" earlier document published on or after the international filing date</td> <td>"Y"</td> <td>documents of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Z"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table> |                                                                                                                                                                                         |                                                                                                                                                                                                                                               | * Special categories of cited documents: | "T" | later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principles or theory underlying the invention | "A" document defining the general state of the art which is not considered to be of particular relevance | "X" | documents of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone | "E" earlier document published on or after the international filing date | "Y" | documents of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art | "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "Z" | document member of the same patent family | "O" document referring to an oral disclosure, use, exhibition or other means |  |  | "P" document published prior to the international filing date but later than the priority date claimed |  |  |
| * Special categories of cited documents:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | "T"                                                                                                                                                                                     | later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principles or theory underlying the invention                                             |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| "A" document defining the general state of the art which is not considered to be of particular relevance                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | "X"                                                                                                                                                                                     | documents of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone                                                                     |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| "E" earlier document published on or after the international filing date                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | "Y"                                                                                                                                                                                     | documents of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | "Z"                                                                                                                                                                                     | document member of the same patent family                                                                                                                                                                                                     |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| "O" document referring to an oral disclosure, use, exhibition or other means                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                         |                                                                                                                                                                                                                                               |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| "P" document published prior to the international filing date but later than the priority date claimed                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                         |                                                                                                                                                                                                                                               |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| Date of the actual completion of the international search<br>06 JULY 1995                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                         | Date of mailing of the international search report<br>19 JUL 1995                                                                                                                                                                             |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                                                                                                                                         | Authorized officer<br>Deborah Crouch, Ph.D. <i>Deborah Crouch</i>                                                                                                                                                                             |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |
| Facsimile No. (703) 305-3230                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                         | Telephone No. (703) 308-0196                                                                                                                                                                                                                  |                                          |     |                                                                                                                                                                                                   |                                                                                                          |     |                                                                                                                                                                           |                                                                          |     |                                                                                                                                                                                                                                               |                                                                                                                                                                        |     |                                           |                                                                              |  |  |                                                                                                        |  |  |